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The exploration of novel symbiotic bacteria that may have influential roles in sponge life history

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ABSTRACT:

Sponges produce an impressive variety of secondary metabolites that perform a variety of ecological functions. Many marine sponges even harbor diverse carotenoid compounds, an unusual class of secondary metabolites that animals are incapable of producing. Furthermore, sponges serve as hosts to an astonishingly diverse microbial community that can occupy up to sixty percent of a sponge's biomass. Our research ultimately hopes to link microbial species to the production of secondary compounds, like carotenoids, and to assess the ecological role of such compounds and their effect on sponge life history strategy. This study describes the ecological distribution of two species of sponge, *Clathria prolifera* and *Halichondria bowerbanki,* from the Chesapeake Bay (Virginia, USA) as well as analyzes their general larval behavior. Culturable and non-culturable microbial communities harbored by both species of sponge were examined. A diverse group of pigmented microbes were isolated and identified as *Roseobacter/ Rhodobacteraceae* sp, *Shewanella* sp, and *Pseudoalteromonas* sp. Our data indicates that the symbiotic bacteria may be producing compounds that influence aspects of the sponge phenotype, behavior, and distributional patterns.

INTRODUCTION:

Marine sponges compose a significant portion of benthic communities throughout the world and have a substantial impact both on benthic community composition and pelagic processes (Gili and Coma, 1998). Sponges are among the oldest multicellular organisms and simplest Metazoans found in all marine systems (Hentschel et al. 2002). They are sessile as adults and despite a simple body plan are successful filter-feeders, capable of obtaining nutrients by removing particulate organic matter or bacteria from surrounding sea water (Hentschel et al. 2002; Pile and Young, 2006). Furthermore, sponges serve as hosts to diverse microbial communities that can occupy up to sixty percent of a sponge's biomass (Santavy et al. 1990).

Microbial-symbiont communities have been found to perform important functions for their sponge host, including but not limited to nutrient cycling and secondary metabolite production (Friedrich et al. 2001). Research on many of these metabolites has lead to the discovery of pharmaceutical compounds containing antimicrobial, cytotoxic,

and antitumor activities (Hentschel et al. 2003). Each year more active metabolites are obtained from sponges then any other marine taxon (Blunt et al. 2006). Despite the apparent importance of sponge-microbe associations little is actually known about the structure, function, and stability of these interactions (Stanton 2003).

Our present understanding of sponge-microbe symbioses is limited at best (Stanton 2003), but it contains the potential to serve as a model system to study the dynamics of host-symbiont relationships. An inability to cultivate many of these microbes has hindered efforts to understand microbial diversity (Maldonado 2007, Taylor et al. 2007). However, in the past several years a variety of molecular non-culture based and culture-based approaches have been developed and used to explore the sponge-microbe relationship (e.g. Devereux and Wilkinson, 2004; Taylor et al. 2007; Webster et al. 2001, 2004; Hentschel et al. 2002, 2003; Hill et al. 2006; Weisz et al. 2007). These studies have greatly enhanced the understanding of microbial diversity in marine sponges as well as provided the means to recognize subtle patterns of host-symbiont distribution. For instance, a new bacterial lineage that is specifically sponge-associated has been identified and termed the *Poribacteria* Phylum (Fieseler et al. 2004). Further, it appears that sponges may harbor both generalist microorganisms as well as specialist (Hill et al. 2006, Schmitt et al. 2007) and may also be classified as either as low microbial abundance (LMA) species or as high microbial abundance (HMA) species (Weisz et al. 2008).

The transmission of microbial symbiont communities across generations has also been studied (Carait et al. 2007; Schmitt et al. 2007; Maldonado 2007). Analysis of electron microscopy provides evidence that transmission of microbial symbionts is vertical and that this mechanism may provide an energetic advantage to the planktonic larvae of the studied sponge species (Carait et al. 2007; Schmitt et al. 2007). Further, Schmitt et al. (2007) proposes the idea that vertical transmission is important in the maintenance of phylogenetically complex (yet sponge-specific) microbial communities. In her study, larvae released by the same *Ircinia felix* adult sponge individual contained highly similar microbial communities (assessed using DGGE), whereas larvae released by different *I. felix* adult individuals showed slightly different microbial communities (different DGGE banding patterns). Vertical transmission of bacterial symbionts appears

to be a specific process in that microorganisms from adult *I. felix* are passed on, but an unselective process in that all bacterial phylogenetic lineages are passed on from adult to larvae (Schmitt et al. 2007).

There is a limited amount of information available on the roles that specific microorganisms play in sponge-microbe associations. In general microbes are involved in nutrient cycling and secondary metabolite production (Friedrich et al. 2001). However, identifying which microbe produces a specific compound is complicated, and even more complicated is linking the presence of a compound or secondary metabolite to sponge host function and behavior. Carotenoids, for instance, are an unusual class of secondary metabolites in animals. Animals are unable to produce carotenoid compounds *de novo,* nonetheless, many marine sponges contain high concentrations of diverse carotenoid compounds in their tissues (Litchfield and Liaaen-Jensen, 1979; Liaaen-Jensen et al. 1982; Margalith 1992; Matsuno 2001; Hill unpublished data). The ultimate origin of these compounds is currently unknown but many suggest that carotenoids could be derived through diet or microbial symbionts. *Clathria prolifera,* a temperate marine sponge, produces high concentrations of diverse carotenoid compounds yet the function of these compounds remains undetermined (Hill unpublished data). There are also several types of bacteria that are known to produce anti-fouling compounds (Holström et al. 1992, 1998; Egan et al. 2000, 2001, 2002; Mai-Prochnow et al. 2004). Such chemical compounds could play a tremendous role in life history patterns, including larval settlement cues and spatial competition of sponges harboring these types of bacterium.

The aim of this research is to ultimately link microbial species to the production of secondary compounds, like carotenoids, and to assess the ecological role of such compounds and their effect on sponge life history strategy. The ecological distribution of two species of sponge, *Clathria prolifera* and *Halichondria bowerbanki,* from the Chesapeake Bay (Virginia, USA) was assessed along with the general larval behavior for these species. This research seeks to relate these aspects of the life history of *C. prolifera* and *H. bowerbanki* to secondary metabolites produced by the sponge or its symbionts. Overall microbial diversity and stability in these two sponge species was also addressed using culture based and non-culture based techniques.

MATERIALS AND METHODS:

Sample Collection

Sponge samples were collected from June-August 2007 from the pier at the Virginia Institute of Marine Science (VIMS) in Gloucester Point, Virginia (USA). Healthy *Clathria prolifera*, *Halichondria bowerbanki,* and *Haliclona loosanoffi* sponges were collected from pier pilings at depths ranging from 0.5 to 2 m at low tide. Collected samples were transported in aerated tanks with filtered sea water to the University of Richmond, and processed within five hours of collection. Small samples of adult sponge tissue were snap-frozen in liquid nitrogen and stored at -80°C until subsequent DNA extraction. This was the general procedure for all sponge collections.

Larvae were collected from each reproductive adult sponge as well. Once back at the University of Richmond adult sponges were kept in separate aerated tanks with filtered sea water. As larvae were released from the adult sponges they were collected using pipettes and pooled for DNA extraction (n=50-100/adult), microbial analysis (n=80-100/species), and behavioral analysis (n=100-150/species). Larvae pooled for DNA extraction or microbial analyses were washed in sterile, filtered sea water at least five times before subsequent analysis.

Adult Distribution Analysis

Substantial colonies of *C. prolifera* and *H. bowerbanki* sponges exist along the pier pilings at VIMS, but the sponge community structure has never been assessed in this area. From June to August 2007 the number and size of adult colonies growing on each pier piling was recorded. The depth of each individual below the lowest low tide marker was recorded as well as the individual's location in respect to the pier itself (aka inner piling or outer piling).

Larval Behavior Analysis

In order to characterize larvae from *C. prolifera*, *H. bowerbanki,* and *H. loosanoffi* and to assess their behavioral patterns larvae were collected (n=100-150) as described above during each sponge's reproductive period (all sponge species were reproductive at some

point from mid-June to early July). Newly released larvae (1-4 h) form each species were collected in the laboratory and pooled into glass dishes containing 10 to 30 ml of filtered sea water. Each dish held batches of 3 to 15 larvae from a particular species. Using a dissecting microscope and a ProgRes C14 microscope camera detailed pictures were taken documenting the size and shape of each larval species and larval swim speed and swim direction (deviation, in degrees, from a straight line) were also analyzed.

Larval swimming speed was estimated by tracing the trajectory path of the larvae after a long exposure picture (1sec). The brightly colored larval species left a smear during the long-exposure pictures that could be easily observed and traced. The distance traveled in one second and deviation, in degrees from a straight line (i.e., 180°), were both measured using Image J software. All pictures used for analysis were taken under the same camera settings at 10x magnification. The proper scale was determined by converting pixels into millimeters (at 10x 78 pixels = 1mm). All measurements for a species were averaged to provide a general characterization for each species. A detailed account of general swimming observations was also recorded. Time laps pictures were taken ranging from 2 to 4.5 seconds with pictures every 100 milliseconds to capture these general larval behavior patterns.

Microbial Analysis

Larvae collected from reproductive adults were pooled in order to identify some of the microbial symbionts closely associated with *C. prolifera* and *H. bowerbanki.* Pooled larvae (n=80-100) were washed in sterile, filtered sea water five times. After the final rinse the larvae were suspended in sterile filtered sea water and crushed/ground. The larval mixture was then mixed in various ratios with sterile filtered sea water to form a 1x, 1:1, 1:10, and 1:100 solution. Solutions were plated on Marine Agar (Difco 2216) and cultures were left upside down for 48 hours. Pigmented bacterial colonies were then selected and purified. Freezer stocks of purified bacteria were created by growing picked colonies in 20% glycerol and marine broth (Zobell 2216).

Microbial analysis included determining gram status and pigment production. Gram negative status was determined visually with potassium hydroxide tests as described by Whitman and MacNair (2004). Bacterial pigment extracts were assessed by

UV/visible light spectroscopy, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) analysis. Pigments produced from each of the bacterial lawns were extracted in acetone for at least one hour and the filtered through a 0.45 μm filter for HPLC analysis. The acetone extracts were also used in TLC analysis with the non-polar 1:1 Ethanol Acetate:Hexane solvent and the polar 1:1 Ethanol Acetate:Methanol solvent. Peak absorbencies for extracted compounds were obtained by UV spectroscopy.

DNA was also extracted from each bacterial isolate using the CTAB protocol (Hill et al. 2004). DNA quality was verified by gel electrophoresis using a 1% agarose gel. DNA was quantified using the Quiagen UV spectrophotometer and diluted to 20ng μl^{-1} The universal bacterial primers 27f and 1492r were used to amplify the bacterial 16s ribosomal subunit from the extracted DNA. The PCR cycling conditions included a 4min at 94°C denaturing step; 30 cycles of 30s at 94°C, 30s at the 50°C annealing temperature, and 45s at 72°C; and one final elongation step for 2min at 72°C. The PCR product (5μl) was run on a 1% agarose gel to verify amplification. Successful amplicons were cut out of the 1% agarose gel and cleaned using the QIA Gel Extraction Kit (Qiagin). Extracts were sent to our colleague Olivia Harriott at Fairfield University to be sequenced.

Bacterial sequences were then phylogentically analyzed. The BLAST algorithm at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) was used to determine the identity of DNA sequences obtained from our bacterial isolates. Sequences from other bacterial species that had a high degree of similarity with our isolate were aligned using ClustalX. Neighbor joining trees (using the minimum evolution optimality criterion) were produced from these DNA alignments using PAUP*B4.1.

Transmission Analysis

C. prolifera release their larvae once a year around mid June. Reproductive individuals were collected from June 15 through June 26 2007 and transported back to the laboratory at the University of Richmond. To assess the transmission of bacterial symbionts from reproductive adult sponges to larvae, larvae were collected as described above and pooled

(n=50-100/reproductive adult). Tissue from the reproductive adults (n=4) was also collected and stored at -80°C until DNA extraction.

Mother and larval DNA was extracted using the MoBio UltraClean® Soil DNA Isolation Kit (MoBio Laboratories, Inc) following the alternative protocol for maximum yields. DNA quality was verified by gel electrophoresis using a 1% agarose gel. Two sets of primers were used to amplify the bacterial 16s ribosomal subunit. The UNI 1055f and 1406r primer set was used to amplify a conserved, universal domain of the 16s ribosomal gene. The V3 PRBA338f and PRUN518r primer set was used to amplify a more variable region of the 16s ribosomal subunit. The thermocycler conditions for the UNI primer set included a 2min at 95°C denaturing step; 35 cycles of 1min at 95°C, 30s at the 57°C annealing temperature, and 45s at 72°C; and one final elongation step for 6min at 72°C. The cycling conditions for the V3 primer set included a 2min at 94°C denaturing step; 3 cycles of 1min at 94°C, 30s at a 54°C annealing temperature, and 1min at 72°C; 35 cycles of 45s at 94°C, 30s at a 63°C annealing temperature, and 30s at 72°C; and one final elongation step for 5min at 72°C. The PCR product (5µl) was run on a 1% agarose gel to verify the quality of amplification.

Denaturing gradient gel electrophoresis (DGGE – BioRad Dcode Universal Mutation Detection System) was used to compare microbial symbiont community structure of mother and larval sponges. The final PCR products (15µl) were run on a 10% polyacrylamide gel containing a linear denaturant gradient ranging from 30 to 60%. The DGGE gel was run at 168V in 1X TAE buffer at 60°C for six hours. The gel was stained with ethidium bromide (0.5 µg ml-1) for 20 minutes, de-stained in 1x TAE buffer for 20 minutes, and then visualized using UV transillumination on a Kodak Gel Logic camera system. Banding patterns were observed and noted, especially similarities and differences among mother vs. larval sponges.

Flow-through Seawater System and Thermal Stress Experiment

A live flow-through seawater system was set up at the VIMS laboratory in Gloucester Point, Virginia (USA). Sea water from the Chesapeake Bay was pumped and filtered through the VIMS aqueduct system into large water tables. A system was constructed in one of the water tables that channeled filtered water via ½'' PVC into six 25 gallon tanks

(Figure 1). Two tanks served as control tanks and seawater was left at its ambient temperature (≈27°C). Submersible heaters where added to the remainder of the tanks and two tanks were heated to +1.5°C from the ambient temperature (≈28.5-29°C) and the final two tanks were heated to +3.0°C from the ambient temperature(≈31-31.5°C). Temperature treatments were randomly placed. Water flow in the system was adjusted to allow the movement of water throughout the system without overwhelming the heating capabilities of the submersible heaters. The temperatures in the system were allowed to stabilize for a few days before the sponge samples were added.

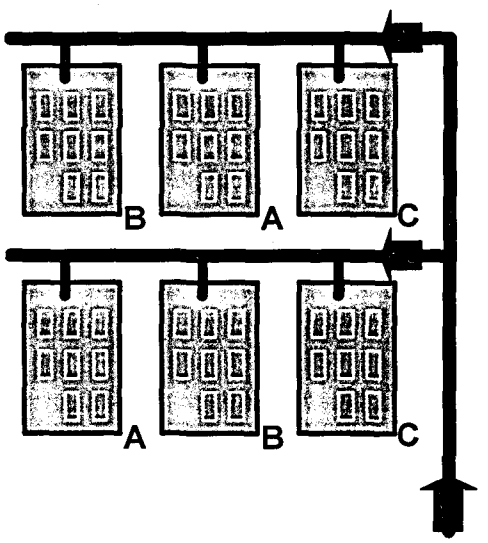

Figure 1: Schematic diagram of the live flow-through seawater system. Letters represent different treatments: A = ambient, B= +1.5°C, C= +3.0°C.

Healthy *C. prolifera* adults (n=16) were collected from the VIMS pier and transported to the live flow-through system. Three small pieces of sponge tissue (about 4cm^2) were cut from each sponge genotype and placed into individual microcosms equipped with holes to allow the circulation of fresh seawater. Microcosms were placed into one of the treatment tanks so that every sponge genotype had a microcosm in each temperature treatment. Temperature treatments had 16 replicates in all with eight sponges per tank and two tanks per treatment. Samples of the sponges were collected as pre-treatment controls and were snap-frozen in liquid nitrogen and stored at -80°C until subsequent analysis. The system was left intact for two weeks during the Chesapeake Bay's thermal maximum and broken down on September 18, 2007. The sponge samples were snap-frozen in liquid nitrogen and stored at -80°C along with the pre-treatment samples until DNA extraction.

Sponge DNA was extracted using the MoBio UltraClean® Soil DNA Isolation Kit (MoBio Laboratories, Inc) following the alternative protocol for maximum yields. Sponge samples from each temperature treatment were pooled for DNA analysis into pre-treatment (n=16), ambient (n=16), and heat stressed (n=29) samplings. The two

temperature treatments (+1.5°C and +3.0°C) were pooled together for the DNA extraction to form one "heat stressed" treatment to be compared with the pre-treatment samples and the ambient seawater samples. DNA quality was verified by gel electrophoresis using a 1% agarose gel. The DNA was treated as described above and amplified using the UNI PCR primer set as well as the V3 PCR primer set. DGGE analysis was also performed using the before mentioned protocol. The DGGE gel was run at 169V in 1X TAE buffer at 60°C for six hours. The gel was stained and visualized using UV transillumination on a Kodak Gel Logic camera system. Banding patterns were compared and contrasted between pre-treatment sponges, ambient temperature sponges, and heat stressed sponges.

RESULTS:

Adult Distribution Analysis

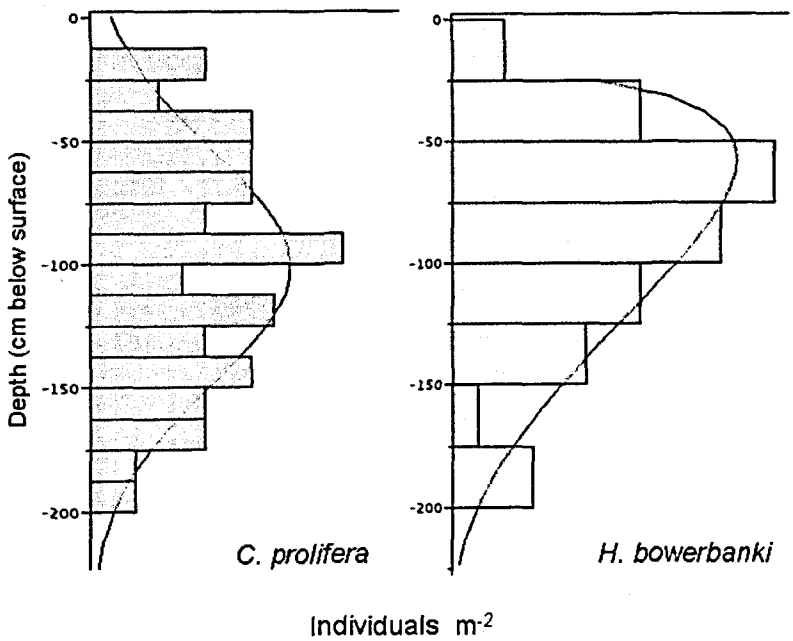

Figure 2: Depth distribution data for *C. prolifera* and *H. bowerbanki* at the VIMS pier. Data collected summer 2007.

Depth distribution of adult *C. prolifera* and *H. bowerbanki* on the pier at Gloucester Point, VA was analyzed (Figure 2). *C. prolifera* exhibits a normal distribution centered around a depth of 1m below the lowest low tide surface. *H. bowerbanki* has a skewed distribution with more individuals located at shallower depths (average depth = 0.8 m).

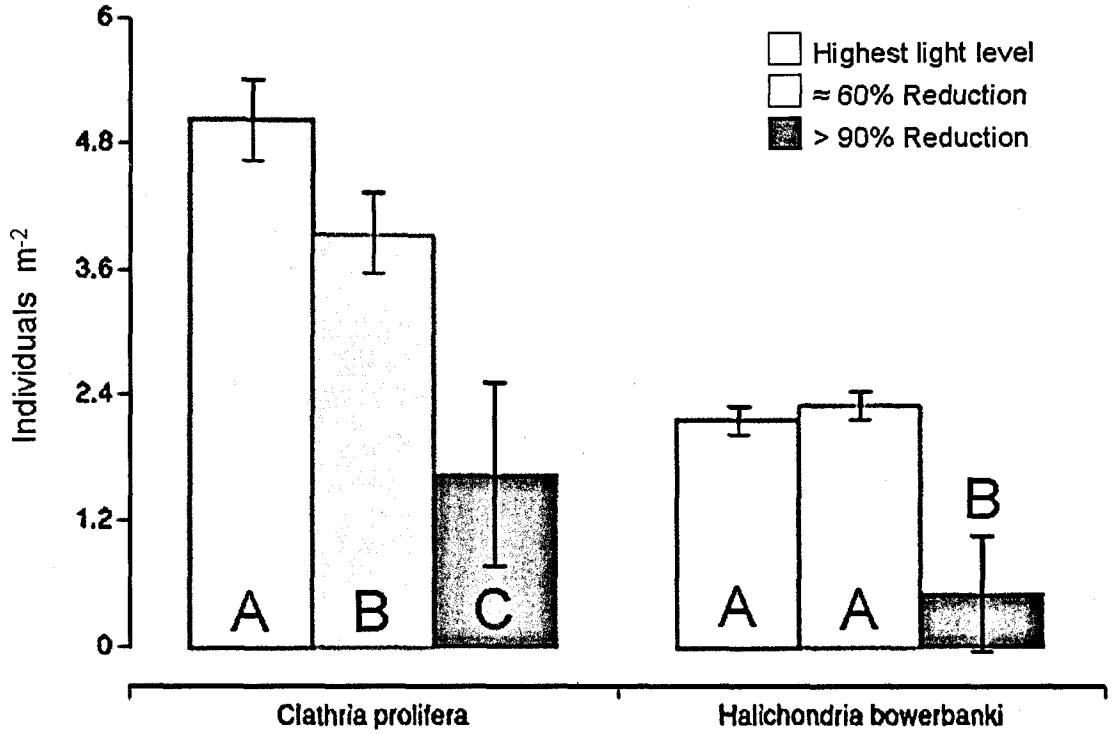

Figure 3: Light level sponge density data for *C. prolifera* and *H. bowerbanki* at the VIMS pier. Data collected summer 2007. Bars not sharing a letter are significantly different - One-way ANOVA, *Cp,* $F_{2,147}=8.4$, $p<0.001$; *Hb*, $F_{2,147}=5.17$, $p<0.01$.

C. prolifera and *H. bowerbanki* sponge distribution was analyzed in relation to light levels along the pier (Figure 3). Highest light levels were found on outer pilings at the VIMS Pier while interior pilings experienced a 60% reduction in ambient light. Light levels at 2 m depth were < 90% that of the surface. Significant differences in average *C. prolifera* and *H. bowerbanki* sponge density were observed (*Cp,* $F_{2, 147}=8.4$, $p<0.001$; *Hb*, $F_{2, 147}=5.17$, $p<0.01$).

Larval Behavior Analysis

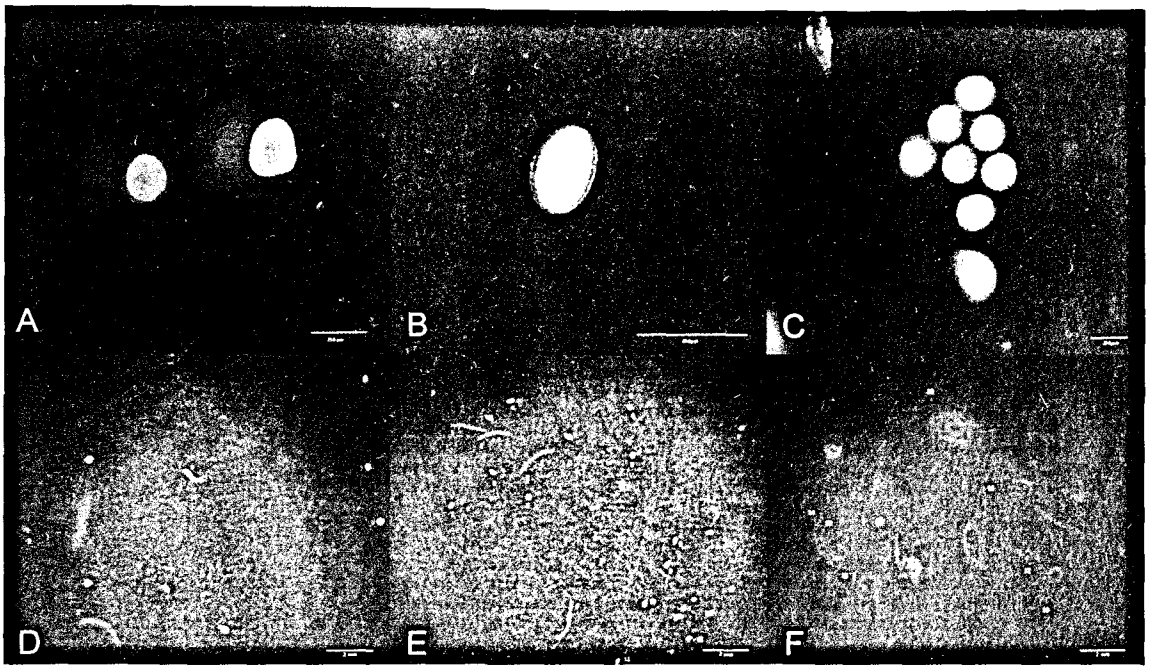

Figure 4: ProgRes C14 microscope camera images of (A) *C. prolifera,* (B) *H. bowerbanki,* and (C) *H. loosanoffi.* Long exposure pictures (1sec) documenting swim speed and direction of (D) *C. prolifera,* (E) *H. bowerbanki,* and (F) *H. loosanoffi.* Bar = 250μm for (A), (B), (C). Bar = 2mm for (D), (E), (F).

Behavioral patterns of larvae from *C. prolifera*, *H. bowerbanki,* and *H. loosanoffi* were assessed shortly after larval release using a ProgRes C14 microscope camera and Image J software (Figure 4). The length and width of each larval species was measured and larval swim speed and swim direction (deviation from a straight line) were also analyzed. *C. prolifera* and *H. loosanoffi* larvae are about the same length, but *C. prolifera* (0.276±0.047 mm long, 0.218±0.038 mm wide) are more rounded in shape while the *H. loosanoffi* (0.277±0.05 mm long, 0.157±0.019 mm wide) are elongated. *H. bowerbanki* larvae were slightly shorter in length then the other larval species (0.219±0.035 mm long, 0.133±0.018 mm wide), but were also elongated in shape. *H. loosanoffi* larvae were the fastest swimmers of the larval species, followed by *C. prolifera* then *H. bowerbanki* (Figure 5). *H. bowerbanki* larvae generally swam in a linear manner while *C. prolifera* larvae swam in a more arc-like motion. *H. loosanoffi* larvae had the greatest diversity in swim direction, often swimming for periods in long straight lines followed by periods of tight twisting and bending (Figure 4F, 5).

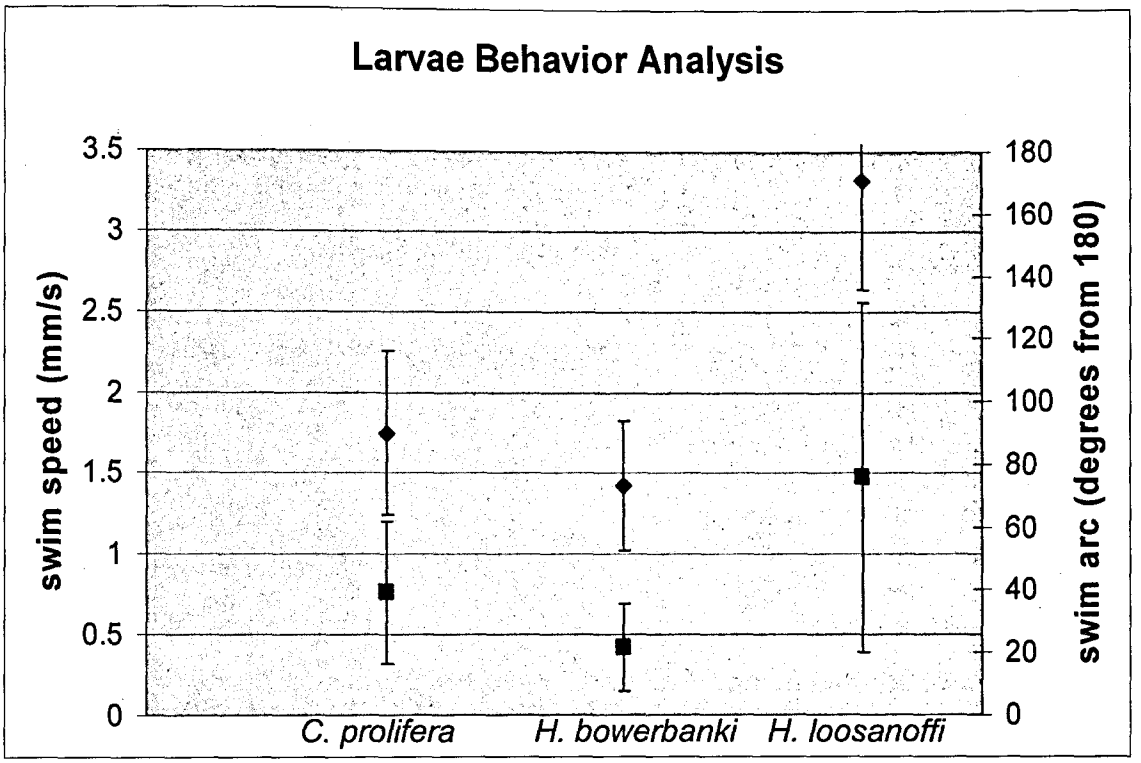

Figure 5: Mean swim speed and swim curvature for *C. prolifera*, *H. bowerbanki*, and *H. loosanoffi* larvae. Data obtained from ProgRes C14 microscope camera and analyzed using Image J software.

A detailed account of general swimming observations for *C. prolifera*, *H. bowerbanki*, and *H. loosanoffi* was also recorded. There appeared to be some evidence that *C. prolifera* and *H. bowerbanki* were photo-sensitive. *C. prolifera* larvae would swim towards the surface of the water and then "bounce" along the top of the water. Likewise, when collecting *H. bowerbanki* larvae with pipettes, they would migrate towards the surface of the water column if a light was placed over the collection tank. In one particular instance larvae were placed in a flask with a desk lamp aimed on the neck of the flask. The *H. bowerbanki* larvae collected in the flask's neck.

Additionally, *C. prolifera* larvae demonstrated three general types of swimming behaviors while being observed in glass dishes containing about 30ml of seawater: directional swimming, crawling, and standing still. During directional swimming the *C. prolifera* larvae would swim in an anterior to posterior orientation by pushing water behind them using their ciliated surface (Figure 4A). The projection forward was arc-shaped and they swam in a corkscrew-like pattern (Figure 4D). The crawling behavior was observed several hours post-release (6-12h) and was characterized by the *C.*

prolifera larvae swimming in small, tight circles. During crawling the larvae typically moved in a clockwise direction, bending to their right. During the 1sec long-exposure pictures this behavioral pattern created visible circular patterns (Figure 6). After crawling for some time the *C. prolifera* larvae would eventually come to a stand still, presumably preparing for settlement.

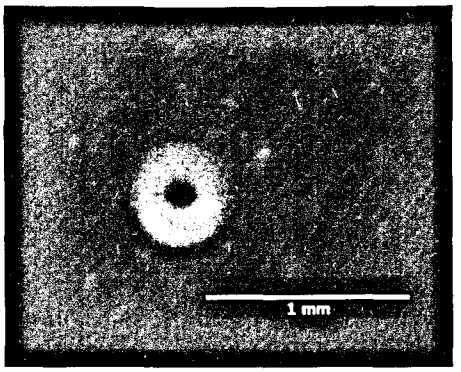

Figure 6: ProgRes C14 microscope camera images of *C. prolifera* exhibiting crawling behavior. Exposure = 1s. Bar = 1mm.

Microbial Analysis

Bacterial species were cultured and isolated from *C. prolifera* and *H. bowerbanki* larvae. Isolates were assigned clone names based on their phenotypic color when grown at room temperature on Marine Agar (Difco 2216). DNA was successfully extracted from seven *C. prolifera* isolates and six *H. bowerbanki* isolates (Table 1). Table 2 summarized their phenotypic coloration and gram status.

Table 1: Phenotypic coloration and gram status for bacterial isolates obtained from *C. prolifera* and *H. bowerbanki* larvae

Source	Isolate	Phenotypic Coloration	Gram Status
Clathria prolifera	Cp101	Red	-
	Cp102	Red	-
	Cp103	Pink	+
	Cp201	Orange	-
	Cp301	Yellow/Brown	+
	Cp401	Green	-
	Cp901	White/Grey	+
Halichondria bowerbanki	Hb101	Pink	-
	Hb301	Orange	-
	Hb302	Yellow/Green	-
	Hb303	Yellow/Brown	-
	Hb304	Yellow	-
	Hb401	Green	-

Colored compounds from the *C. prolifera* and *H. bowerbanki* bacterial isolates were analyzed by extracting compounds from bacterial colonies in acetone. UV spectroscopy analysis was performed on these acetone extracts. The wavelength that had the maximum absorbance was recorded for several of the extracts (Table 2).

Table 2: UV spectroscopy analysis on acetone extracts from *C. prolifera* and *H. bowerbanki* bacterial isolates

Source	Isolate	Max. Absorbance (Au)	Wavelength (nm)
Clathria prolifera	Cp102	0.14395189	532
	Cp201	0.40080118	371
	Cp401	0.13381624	424
Halichondria bowerbanki	Hb301	0.10498714	371
	Hb302	0.04911613	454
	Hb304	0.09187222	368
	Hb401	0.02791595	425

The acetone extracts from the *C. prolifera* isolates were also analyzed with TLC. Several of the isolates, including Cp101 and Cp102, appeared to have non-polar colored compounds that migrated with the 1:1 Ethanol Acetate:Hexane solvent. The red bands that migrated in Cp101 and Cp102 fluoresced under UV light (image not shown). Other bacterial isolates had polar colored compounds that migrated during TLC analysis with the 1:1 Ethanol Acetate:Methanol solvent. The Cp201 and Cp401 extracts migrated and also fluoresced yellow and blue respectively under UV light. HPLC analysis was then run on the acetone extracts from the *C. prolifera* and *H. bowerbanki* bacterial isolates (Figure 7, 8).

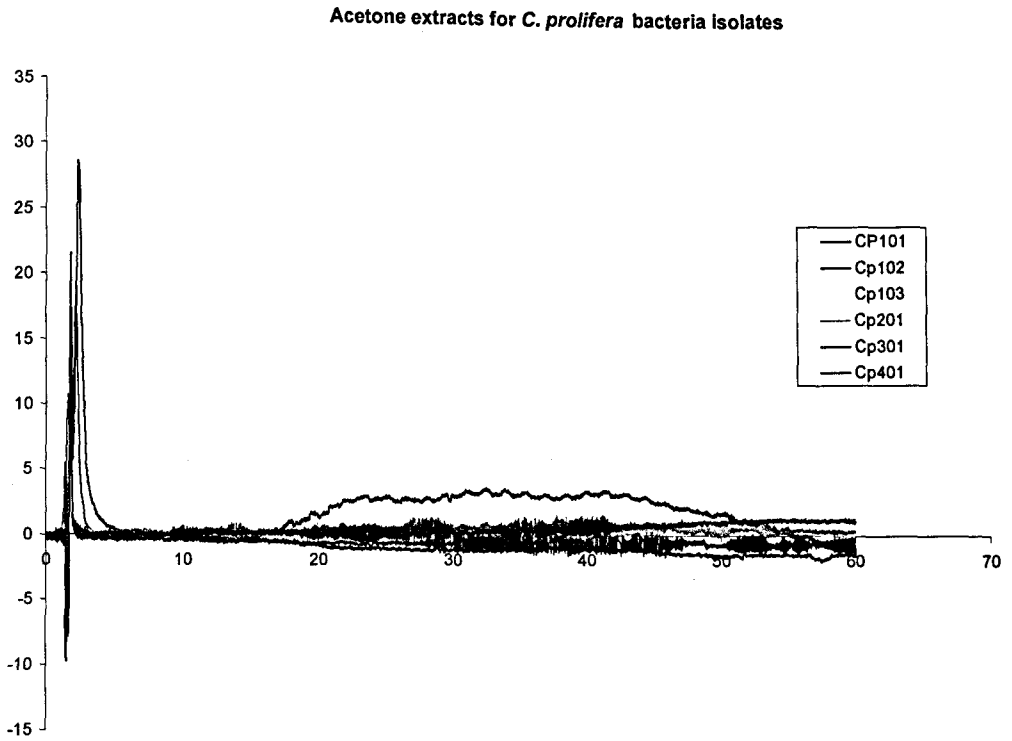

Figure 7: HPLC analysis on acetone extracts from *C. prolifera* bacterial isolates

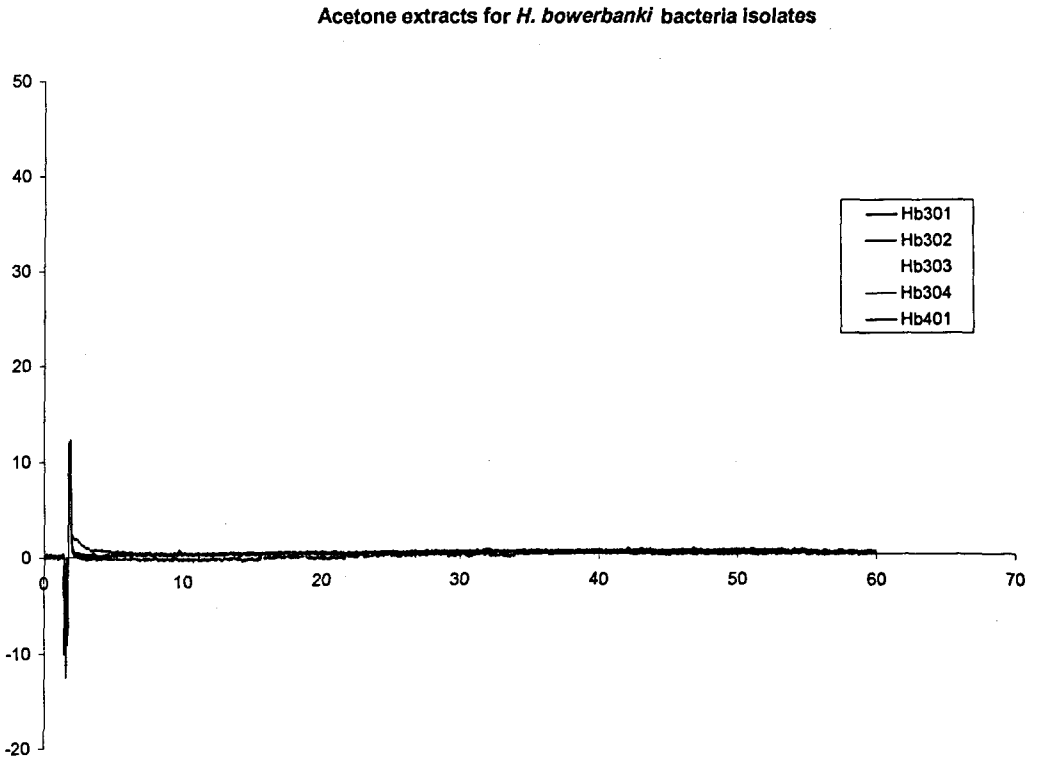

Figure 8: HPLC analysis on acetone extracts from *H. bowerbanki* bacterial isolates

Sequences obtained from DNA extractions on the bacterial isolate listed in Table 1 were phylogentically analyzed. The nearest matches and phylum representatives for the bacterial isolates were identified using the BLAST algorithm at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sequences from other bacterial species that had a high degree of similarity with our isolate were aligned using ClustalX. Neighbor joining trees (using the minimum evolution optimality criterion) were produced from these DNA alignments using PAUP*B4.1. The bacterial isolates clustered into three major lineages: *Roseobacter/Rhodobacteraceae*, *Shewanella*, and *Pseudoalteromonas* (Figure 9, 10, 11).

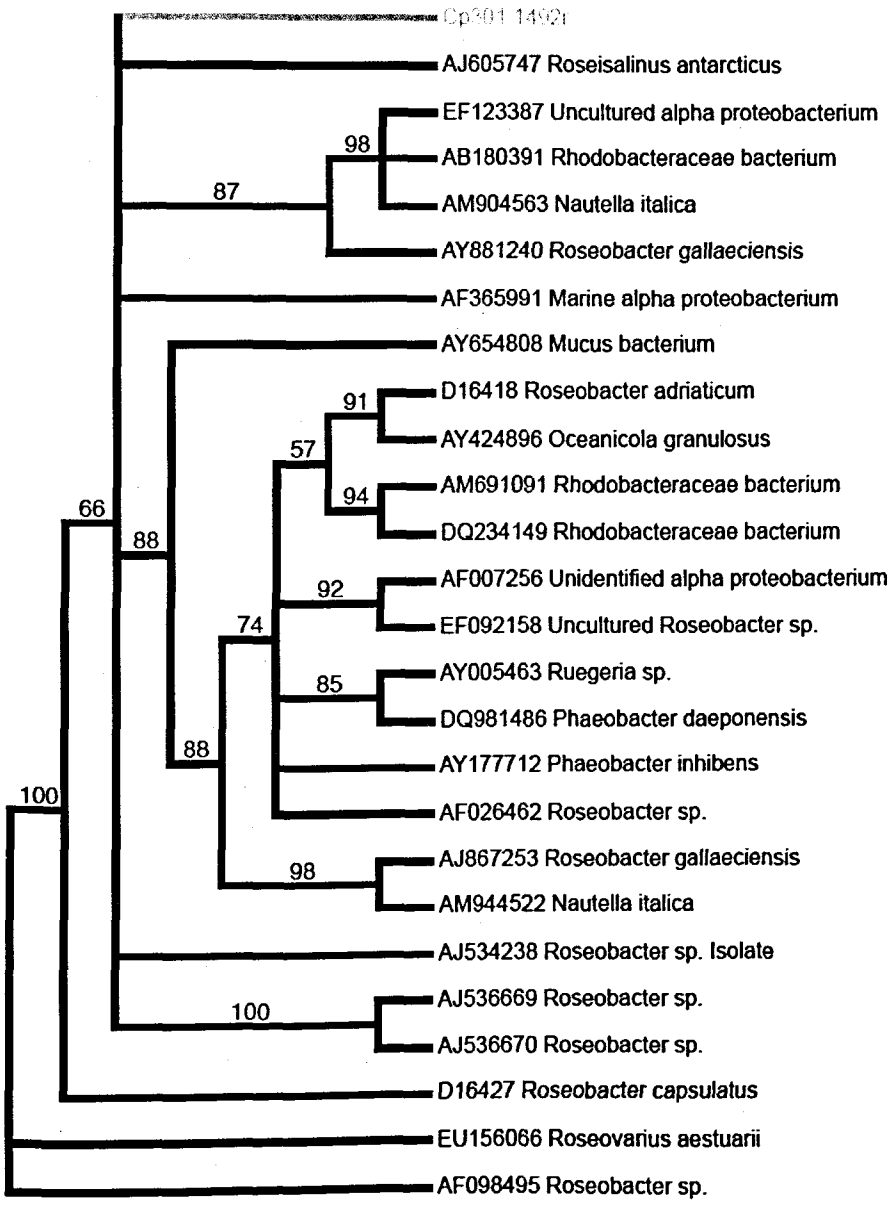

Figure 9: Un-rooted neighbor joining phylogenetic tree with bootstrap support for the *Roseobacter/Rhodobacteraceae* phylum. Sequences obtained from larval isolates are highlighted and the clade they fall in is boxed in yellow.

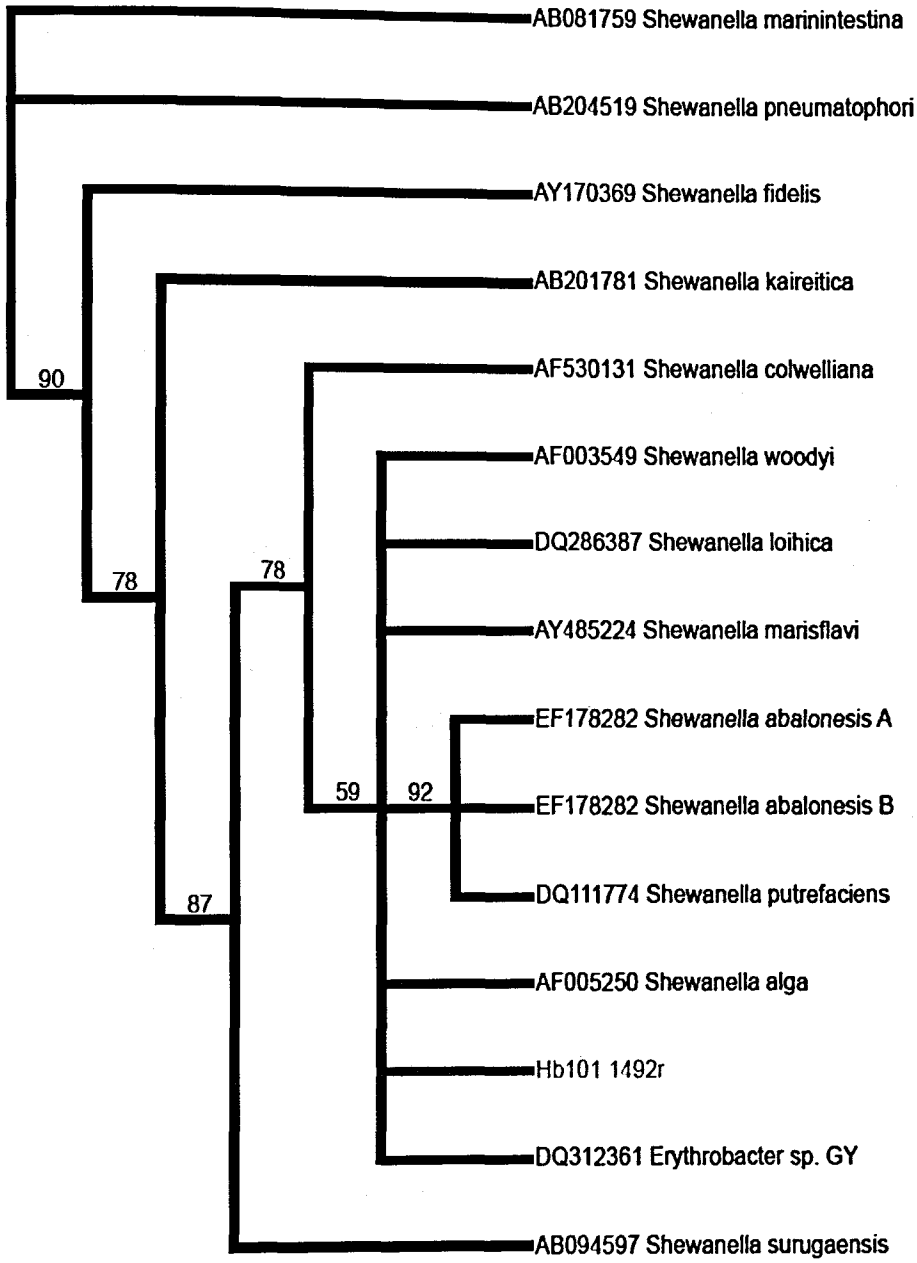

Figure 10: Un-rooted neighbor joining phylogenetic tree with bootstrap support for the *Shewanella* phylum. Sequences obtained from larval isolates are highlighted and the clade they fall in is boxed in pink.

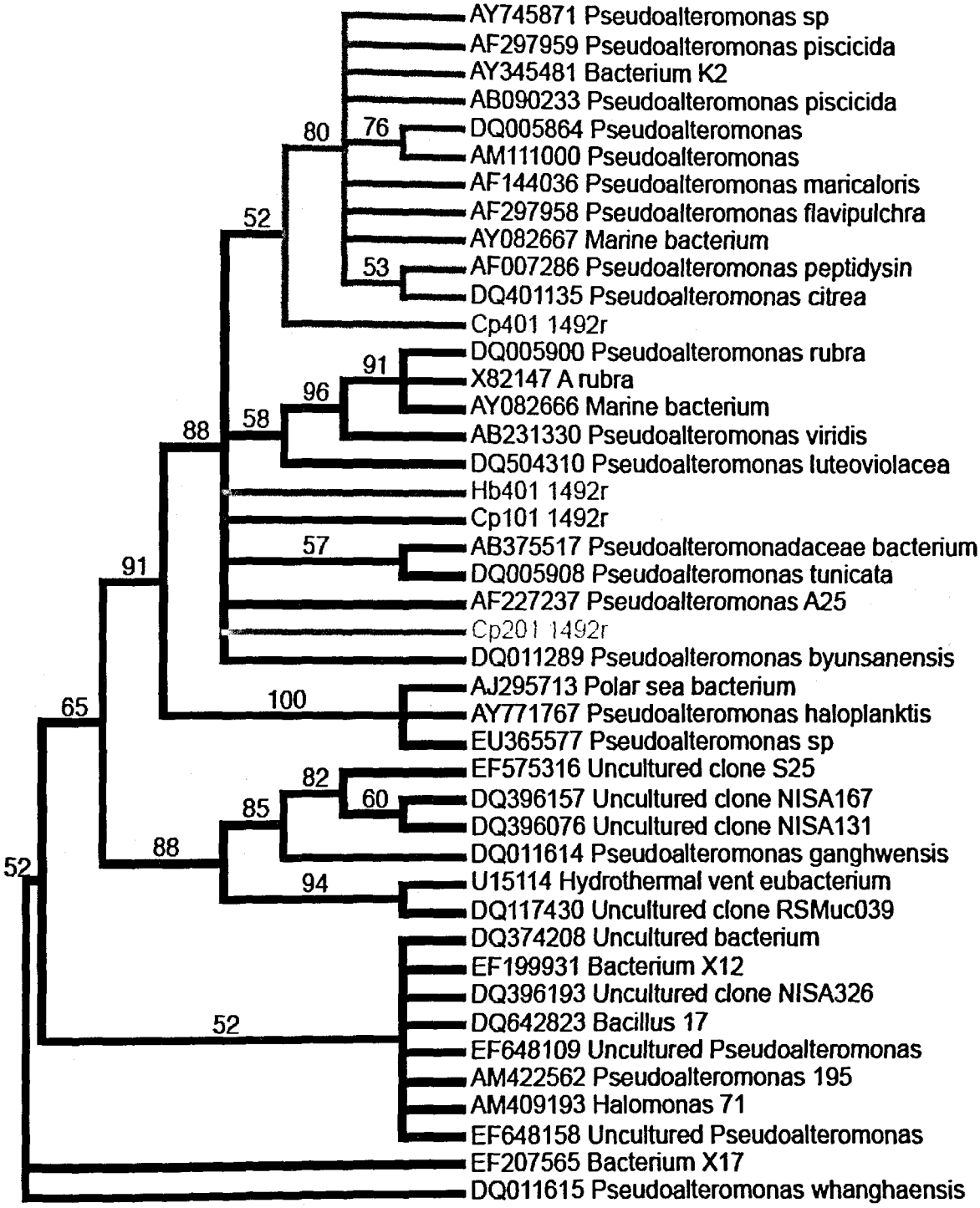

Figure 11: Un-rooted neighbor joining phylogenetic tree with bootstrap support for the *Pseudoalteromonas* phylum. Sequences obtained from larval isolates are highlighted and the clade they fall in is boxed in orange.

Transmission Analysis

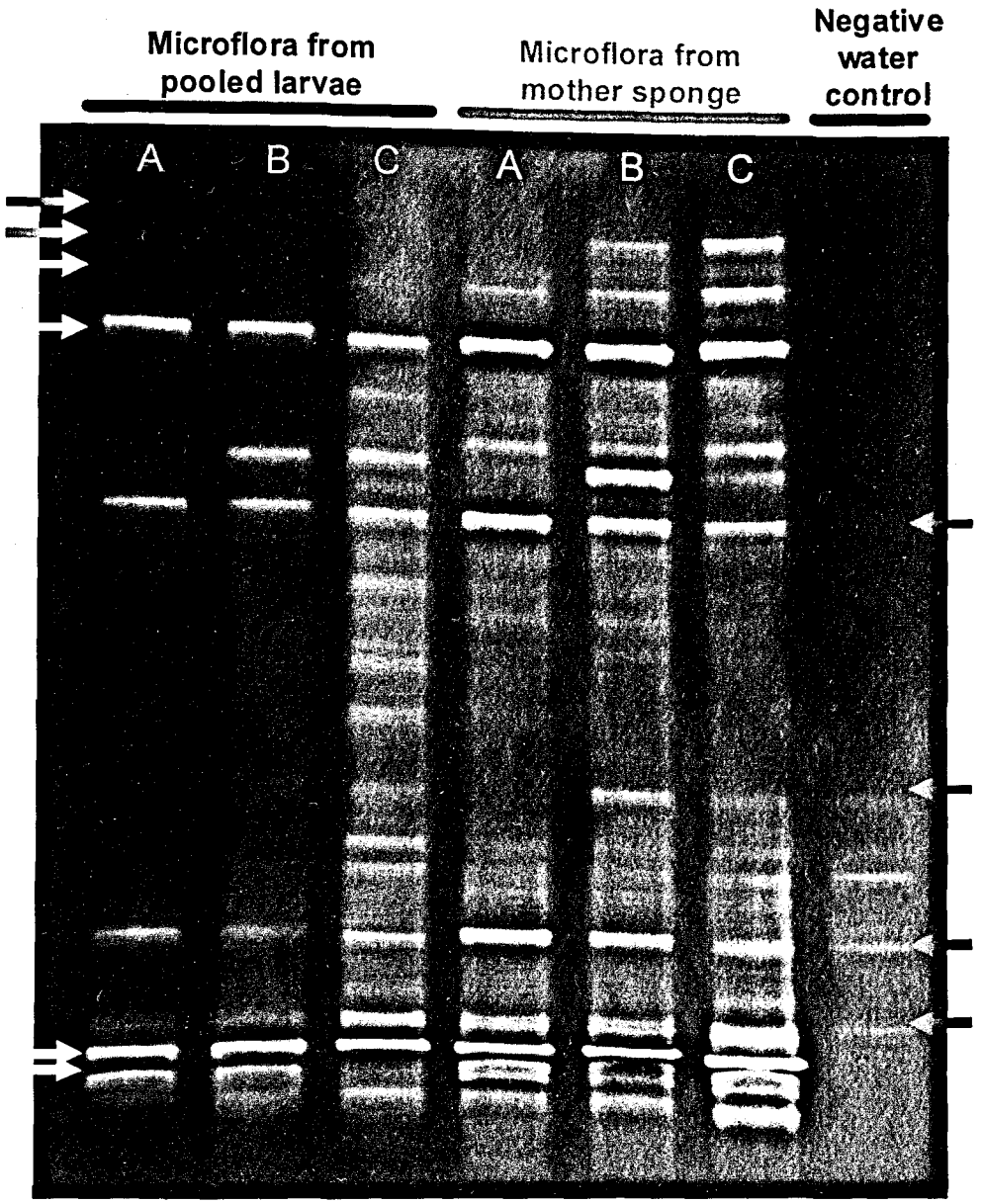

Figure 12: *Clathria prolifera* microflora profiles from mother and larvae DNA sources using V3 primers specific to the bacterial 16s ribosomal subunit (UNI data not shown). Yellow arrows show bands present in both stages, orange arrows show bands present in the mother stage but not the larval stage, red arrows show bands that became more prominent in the larval stage, and blue arrows show bands present in the negative PCR water control. Like-letters represent mother-larval pairs.

To compare microbial symbiont community structure of mother and larval sponges DNA was extracted from reproductive mother sponges and their subsequent larvae. The DGGE profiles from the mother and larvae microflora symbionts were compared using a

30 to 60% denaturing gradient (Figure 12). Multiple bands were observed in both the mother and larval samples. While the majority of bands that were present in the mother also appeared in the larvae, there are some bands that did not appear to be transmitted from the mother sponge to the larvae, and still other bands that appeared more prominent in the larvae. Water contamination during PCR was a problem during this entire process.

Flow-through Seawater System and Thermal Stress Experiment

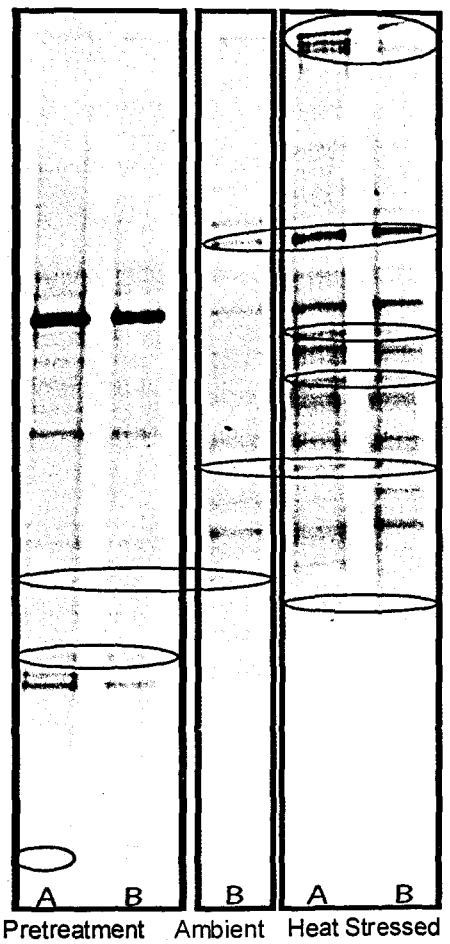

Figure 13: *Clathria prolifera* bacterial profiles with V3 primers from the temperature experiment (UNI data not shown). Pre-treatment (source) sponges, ambient temperature treatments, and heat stressed treatments are designated accordingly. Several bands appeared under heated conditions (circled in purple) and several bands disappeared under heat stress (circled in blue). Bands appeared during the experiment (circled in pink) or disappeared (circled in blue) when compared to the pre-treatment sponges.

To compare microbial symbiont community structure of heat stressed sponges, DNA extracted from heat stressed and non-heat stressed sponges was analyzed using DGGE. The DGGE profiles of symbiont communities from the pre-treatment, ambient, and heat stressed sponges were compared using a 30 to 60% denaturing gradient (Figure 13). The DGGE profile of the temperature experiment showed a number of bands that were present across all treatments. Several bands appeared under heated conditions, while

some bands disappeared after being heat stressed. However, the live flow-through seawater system seemed to also affect the microbial consortia hosted by the sponge as several microbial species appeared or disappeared between the pretreatment samples and the ambient and heat stressed samples. Water contamination during PCR was a problem during this entire process.

DISCUSSION

This study demonstrates that intriguing interactions exist between a sponge and the bacterial symbionts it hosts. Microbial communities in sponges are clearly present in adult and juvenile stages of the *C. prolifera* life cycle (Figure 12) and they appear to be vertically transmitted from one generation to the next. However, little is actually understood about the stability or the purpose of these interactions. That said, the *C. prolifera* and *H. bowerbanki* adult sponges exhibit non-random distributions (Figure 2) that suggest there is some sort of behavioral response to environmental factors or interaction affecting these sponges. This research began analysis on microbial species to identify and characterize the partners involved in the sponge-microbe symbiosis.

In recent years new molecular and culture-based approaches have been developed and used to explore the sponge-microbe relationship (Webster et al. 2001, 2004; Hentschel et al. 2002, 2003; Hill et al. 2006; Weisz et al. 2007). Such studies have improved the understanding of microbial diversity in marine sponges. The transmission of microbial symbiont communities across generations has also been studied (Schmitt et al. 2007; Maldonado 2007) and was further explored in *C. prolifera* during this study. Although PCR contamination in the negative water control appeared to be a difficulty throughout the entire process, results demonstrated that a greater diversity of bacteria is present in both adult and larvae sponge versus the water control (Figure 12). When subtracting the water contamination "noise" it was observed that some bacterial species were present in both the adult and larval stage. However, some bacterial species were present in the adults but not the larvae suggesting that they were not passed onto the sponge offspring. On the other hand, some bacterial species became more prominent in the larval stage suggesting that they were preferentially vertically transmitted. Future

research will focus on identifying the species propagated from one generation to the next by excising DGGE bands of interest and sequencing the partial bacterial isolates.

Traditional laboratory techniques were used in this study to begin preliminary identification of bacterial species symbiotically related to the larvae of *C. prolifera* and *H. bowerbanki* sponges. Isolated bacterial species pigment production was analyzed and several of the isolates appeared to have interesting characteristic wavelengths corresponding to their phenotypic coloration. This data as well as the HPLC data indicate that the bacterial isolates with red and orange phenotypes do not appear to contain carotenoid compounds. More then 500 different molecules of the carotenoid family have been described and beta-carotene is usually the major carotenoid pigment with an absorbance at 463 nm (Margalith 1992). Carotenoids typically produce three absorbance peeks ranging from 430-490 nm. Most of the bacterial isolates in this study produced extracts that do not follow this pattern, however, it is possible that the acetone extracts from the Cp102 (λ_{max}=532 nm, second peak at λ=498 nm) and Hb302 (λ_{max}=454 nm, secondary peaks at λ=428, 481 nm) isolates contain carotenoids (Table 3).

Carotenoids are produced by all photosynthetic plants and are thought to play roles in light harvesting in photosynthetic reaction centers and in the extension of the light absorption spectrum to wavelengths not able to be absorbed by chlorophylls. This function, however, is not applicable in non-photosynthetic organisms. Carotenoids have also been linked to photoprotective mechanisms. Carotenoid pigments show a protective role especially related to the damage caused by irradiation by visible light (Griffiths et al. 1955; Hairston 1976; Litchfield and Liaaen-Jensen, 1979; Liaaen-Jensen et al. 1982; Margalith 1992). *C. prolifera* and many other marine sponges produce high concentrations of diverse carotenoid compounds that show signs of seasonal fluctuations (Hill, unpublished data). It is hypothesized that carotenoids may protect these sponges from environmental stressors such as UV radiation or temperature stress. If there is truth to this hypothesis the presence of carotenoids in *C. prolifera* and *H. bowerbanki* sponges could be a powerful explanatory tool for describing their natural distribution in the upper water column and in areas with higher irradiance (Figure 2, 3). Further, the larvae of *C. prolifera* and *H. bowerbanki* sponges appear to be photosensitive, actually swimming towards light. The presence of carotenoids in sponges may serve a photoprotective role

that allow sponge larvae to settle and occupy areas that many other marine sessile organisms must avoid due to radiation and UV stress. Additional behavioral and microbial analyses are needed to further address these ideas.

The DNA sequenced from the bacterial isolates grouped the sponge microbes into three major bacterial lineages: *Pseudoalteromonas*, *Roseobacter*, and *Shewanella*. *Roseobacter* species of bacteria have been found to engage in antagonistic interactions with other marine bacteria and to assist with photosynthesis in stressful marine habitats (Long and Azam, 2001; Allgaier et al. 2003; Labrenz et al. 2005). Many groups are interested in *Shewanella* bacterial species because they have been found in a variety of marine ecosystems as well as in the guts of other marine organisms (Makemson et al. 1997; Miyazaki et al. 2006; Kim et al. 2007). Some of the species produce iron-reducing, oil-degrading, or acid-producing compounds. These functions have caught the interest of industrial enegery groups around the world (Satomi et al. 2003; Roh et al. 2006). *Pseudoalteromonas* are an interesting genus of bacteria, in which several species have been found to produce target-specific inhibitory compounds against other bacteria, algae, fungi, and invertebrate larvae (Holström et al. 1992, 1998; Egan et al. 2000, 2001, 2002; Mai-Prochnow et al. 2004). *Pseudoalteromonas* species are isolated from marine environments around the world and are often found in association with eukaryotic hosts and their surfaces (Holström et al. 1998; Egan et al. 2000).

Should the bacterial isolates cultured from *C. prolifera* and *H. bowerbanki* sponge larvae be related to *Pseudoalteromonas* species that produce antifouling compounds, the information could be used to explain several life history traits in these two sponge species. In marine ecosystems where space is often a limiting factor, it is impressive that these sponges' surfaces remain free from overgrowth of other sessile organisms like algae, tunicates, or other bacteria. The presence of bio-controlling compounds produced by a *Pseudoalteromonas* bacterium could be one preventative mechanism employed by these host sponges. More research in this area needs to be conducted to evaluate such hypotheses, including larvae behavioral and preference analysis and in-depth compound analysis on the cultured bacterial isolates.

While powerful tools have been developed to understand the diversity of microbial species involved in sponge-symbiont associates, many questions still remain

unanswered. This research sought to begin identifying and characterizing specific microbes involved in the symbiosis. The study provides intriguing preliminary results that lead to the formation of even more unanswered questions. The sponge-microbe associations are complex and intricate systems that need to be further studied.

Whole system diversity and stability of the sponge-symbiont interaction needs to be assessed as well, especially under conditions of environmental stress. The thermal stress experiment needs to be re-run and accurately analyzed without PCR water contamination. There is a growing concern regarding global warming and sea surface temperatures are anticipated to increase by about 0.2°C (McCarthy et al. 2001; Thomas et al. 2004). Models predict over the next 50-100 years marine ecosystems will experience an increase in the frequency of stressful thermal events, so understanding the structure and stability of the sponge-microbe symbiosis could prove to be a useful tool in understanding the effects that such changes will have on the marine environment.

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